

Hydrolysis of Adenosine Triphosphate by Crystalline Yeast Pyrophosphatase

Effect of zinc and magnesium ions

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ABSTRACT Schlesinger and Coon's report that crystalline yeast inorganic pyrophosphatase, in addition to its known ability to hydrolyze inorganic pyrophosphate in the presence of Mg ions, is also able to catalyze the hydrolysis of ATP and ADP in the presence of Zn ions was confirmed. A systematic study showed that the ratio of 370 of PPase-Mg over ATPase-Zn activities per milligram protein in various preparations of pyrophosphatase obtained in the course of isolation of crystalline pyrophosphatase from baker's yeast was nearly identical in all the preparations, independent of their purity. The course of hydrolysis of ATP by crystalline pyrophosphatase in the presence of Zn was carried out with the aid of ion exchange on Dowex 1. The finding of Schlesinger and Coon that the hydrolysis proceeds from ATP to ADP and then slowly to AMP was confirmed. The kinetics of the first phase of the reaction was found to depend on the molar ratio of Zn/ATP in the reaction mixture. Mg ions in the presence of Zn ions have an accelerating effect on the rate of hydrolysis of ATP. This suggests strongly that both activities—ATPase and PPase—are manifestations of the same active group in the protein molecule of crystalline pyrophosphatase.

Crystalline yeast pyrophosphatase isolated in 1952 (1) was found to act as a powerful catalyst for the hydrolysis of sodium or potassium pyrophosphate into orthophosphate ions in the presence of magnesium ions, and also to a lesser degree in the presence of other metallic ions such as Mn^{++} or Co^{++} . On the other hand, organic pyrophosphates such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP) were not affected by comparative concentrations of crystalline pyrophosphatase, either in the presence or in the absence of Mg^{++} ions.

In a recent publication, however, Schlesinger and Coon (2) reported that ATP and several other organic pyrophosphates were hydrolyzed by relatively high concentrations of crystalline pyrophosphatase provided magnesium ions

were replaced by zinc ions in the reaction mixture; also, manganous and cobaltous ions could partially be substituted for zinc in effecting ATP or ADP hydrolysis.

The same authors obtained evidence "that the hydrolysis of ATP occurs primarily between the β - and γ -phosphate groups to yield ADP which is then converted to AMP. The hydrolysis of ATP did not occur when Mg^{++} was substituted for Zn^{++} ." The ratios of the two activities, PPase-Mg *vs.* ATPase-Zn of one crude and four crystalline pyrophosphatase preparations were practically identical. Also, partial denaturation by acetone or heat brought about the same percentage loss in both enzymic activities.

The studies of Schlesinger and Coon suggest strongly that the ATPase-Zn activity is not due to an impurity but that the two enzymic activities are carried by one protein.

The studies of Schlesinger and Coon were extended in the present work to the following:

1. Detailed studies were made of the ratios of the specific activities of PPase-Mg to ATP-Zn in various preparations obtained in this laboratory in the course of isolation and crystallization of pyrophosphatase from baker's yeast (3) and in fractions of crystalline pyrophosphatase separated by chromatography with diethylaminoethyl (DEAE) cellulose.
2. Studies of the course of hydrolysis of ATP by crystalline pyrophosphatase were carried out by means of ion exchange chromatography on Dowex I columns.
3. Effect of the concentration of Zn ions relative to that of ATP on the rate of hydrolysis of ATP by crystalline pyrophosphatase.
4. Effect of Mg ions on the rate of hydrolysis of ATP by crystalline pyrophosphatase in the presence of Zn ions.

EXPERIMENTAL TECHNIQUE

1. *Measurement of ATPase and PPase Activities*

(a) ATP hydrolysis mixture at 30°C

- 4 ml 0.1 M ammonium acetate pH 7.0
- 1 ml 0.02 M Na_2ATP (adjusted with NH_4OH to pH 7.0)
- 1 ml 0.01 M $ZnCl_2$
- 1 ml 0.02 M NH_4 acetate containing about 40 micrograms pyrophosphatase

1 ml samples pipetted at various times, usually after 20, 40, and 60 minutes, into 15 ml volumetric flasks containing 1 ml 1 N H_2SO_4 and 5 ml H_2O for inorganic phosphorus determination by the method of Fiske and SubbaRow (4). The ATPase activity, expressed in terms of milligrams inorganic phosphorus liberated per minute at 30°C, is multiplied by 7 (volume of reaction mixture), then by the dilution of the stock of enzyme and divided by stock enzyme concentration (about 0.2 mg per ml 0.02 M NH_4 acetate), thus yielding the specific ATPase activity at 30°C of the sample of enzyme used.

The concentration of enzyme was expressed in terms of the optical density at 280 $m\mu$ of the clearly centrifuged stock solution, divided by 1.5. The protein concentration of crude preparations of the enzyme was determined turbidimetrically by means of CCl_3COOH (1).

(b) $\text{Na}_4\text{P}_2\text{O}_7$ hydrolysis mixture at 30°C

4 ml 0.1 M NH_4 acetate pH 7.0

1 ml 0.01 M $\text{Na}_4\text{P}_2\text{O}_7$

1 ml 0.01 M MgCl_2

1 ml 0.02 M NH_4 acetate solution containing about 0.10 μg pyrophosphatase

1 ml samples taken at various times for inorganic P as described above.

2. *Chromatography of Crystalline Pyrophosphatase on DEAE Cellulose at 5°C (5-7)*

Carried out by Paul T. Englund, Graduate Fellow at The Rockefeller Institute.

The adsorbent, Schleicher and Schuell No. 70 standard ion exchange DEAE cellulose, was washed alternately with 1.0 N NaOH and 1.0 N HCl until the optical density of the washing solution at 280 $m\mu$ was nearly zero. The adsorbent was then equilibrated at 5°C with 0.01 ammonium acetate and packed to a height of 20 cm in a column 0.9 cm in diameter. Nitrogen pressure of 10 pounds per square inch was used to pack the slurry into the column. About 8 mg of twice crystallized pyrophosphatase dissolved in 1 ml of 0.01 M ammonium acetate was placed on the column. The eluting system consisted of a constant volume mixing flask provided with a magnetic stirrer and containing 500 ml 0.01 M ammonium acetate to which 0.5 M ammonium acetate was added continuously at the rate of 3 ml per 12 minutes, equal to the rate of outflow of the effluent into tubes in the fraction collector. This required a hydrostatic pressure of about 70 cm. The effluent samples were analyzed for protein content (optical density at 280 $m\mu$) and also for ATPase-Zn and PPase-Mg activities.

3. *Separation of the Adenosine Phosphates ATP, ADP, and AMP by Ion Exchange on Dowex 1 Column* The method of Cohn and Carter (8) as modified by Siekevitz and Potter (9) was used.

Samples of 1 ml of ATP-Zn-pyrophosphatase hydrolysis mixture pipetted at various times into 10 ml tubes were boiled for 30 seconds to stop the enzymatic reaction, then cooled to 5° and mixed with 3 ml H_2O and 1 ml 0.5 N NH_4OH . The solutions were then put through columns of washed 200 to 400 mesh Dowex 1 (0.8 sq cm \times 2 cm) followed by 20 ml H_2O , and then in succession by a total of 125 to 150 ml in 25 ml portions of the following solutions: (a) 0.003 N HCl for the elution of AMP, (b) 0.01 N HCl-0.02 M NaCl for the elution of ADP, (c) 0.01 N HCl-0.2 M NaCl for the elution of ATP.

The concentration of the adenosine phosphates in the various effluent solutions was determined by measuring their optical densities at 260 $m\mu$, the density at 260 $m\mu$ of 1 micromole adenosine per ml being equal to 14.

EXPERIMENTAL RESULTS

1. *The Ratios of the Specific Activity of Pyrophosphatase-Mg Activity (PPase-Mg) to that of ATPase-Zn Activity in Various Preparations Obtained in the Course of*

Isolation of Crystalline Pyrophosphatase from Baker's Yeast Table I contains a list of six preparations beginning with the crudest first yeast extract fraction precipitated in 0.7 saturation of ammonium sulfate and ending with a preparation of 5 times recrystallized pyrophosphatase, the range of specific PPase-Mg activity extending from 0.72 to 39.5. The ratios of PPase-Mg to the ATPase-Zn activities per milligram protein do not show any trace of a drift on purification of the enzyme, thus proving that the ATPase activity is not due to an accidental impurity. The approximate ratio of 370 was found to hold true for a large number of crystalline preparations obtained from various stocks of commercial baker's yeast.

TABLE I
SPECIFIC ACTIVITIES OF VARIOUS
PREPARATIONS OF PYROPHOSPHATASE

Preparation	Specific activities		Ratios
	PPase-Mg	ATPase-Zn	
1st 0.7.S.A.S.	0.72	0.00204	350
Autolyzed 4 hrs. at 30°C	1.46	0.0044	335
2nd 0.7 s.a.s.	1.86	0.00485	380
1st crystals	17.8	0.045	397
2nd crystals	27.4	0.068	405
6th crystals	39.5	0.112	356
Mean			372

2. *Fractionation of Crystalline Pyrophosphatase by Chromatography on DEAE Cellulose* (See *Experimental Technique*, Section 2) A sample of twice crystallized pyrophosphatase was chromatographed with diethylamino ethyl cellulose. The results of analysis of the various fractions, expressed in per cent of the maximum values of optical density at 280 m μ , of PPase-Mg activity, and of ATPase-Zn activity, per milliliter solution are shown in Fig. 1. It is evident that the sample used contained in addition to the main component also a small amount of an impure component of lower specific activity. The curves for the two enzymic activities, however, coincide throughout the series of samples analyzed, thus proving that the ratios of the two activities are independent of the purity of the components.

3. *The Course of Hydrolysis of ATP by Crystalline Pyrophosphatase in the Presence of Zn* The hydrolysis of ATP was traced conveniently by separating the products of hydrolysis with the aid of ion exchange on Dowex 1 columns (see *Experimental Technique*, section 3). The results are given in Fig. 2.

Phase 1 There is a rapid drop in concentration of ATP accompanied by an immediate appearance and rapid rise in the concentration of ADP.

Phase 2 The ADP formed is gradually degraded to AMP and inorganic

phosphate so that the curve for the formation of ADP shows an initial rapid rise and then a gradual drop in height. The amount of inorganic phosphate liberated during the reaction was found to check closely with the theoretical value of $P = 2 \text{ AMP} + \text{ADP}$.

4. *The Kinetics of ATP Hydrolysis by Crystalline Pyrophosphatase in the Presence of Zn Ions* It has been definitely established by Schlesinger and Coon (2) and also confirmed here, that the hydrolysis of ATP by crystalline pyrophosphatase in the presence of Zn consists of two consecutive phases, namely:

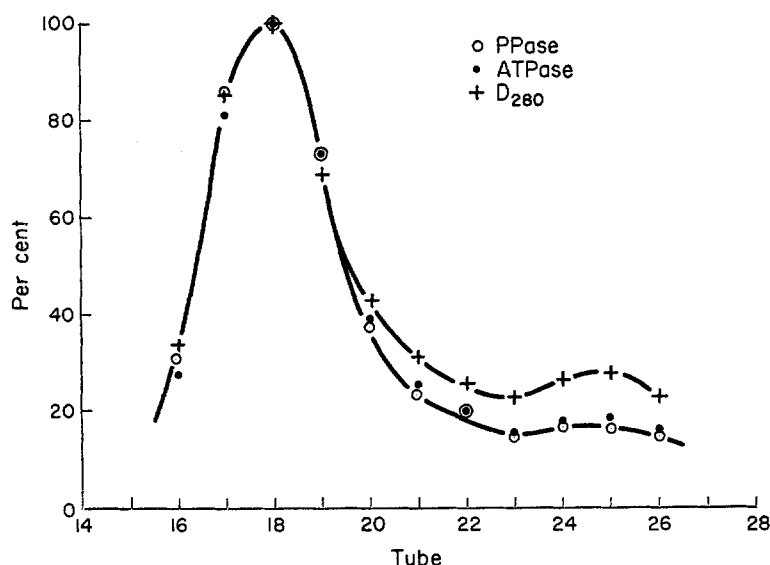
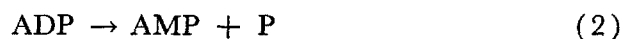


FIGURE 1. Chromatography of crystalline yeast pyrophosphatase on DEAE cellulose.

The first phase of hydrolysis proceeds at a considerably greater speed than the second phase. A mathematical treatment of the kinetics of the whole process of hydrolysis is complicated by the fact that in addition to being dependent on the concentration of enzyme and of substrate, the rate of hydrolysis depends greatly on the concentration of zinc ions in the reaction mixture. The effect on the kinetics of hydrolysis of varying the concentration of Zn ions at constant ATP concentration is shown in Fig. 3 while the effect of varying ATP at constant Zn ion concentration is shown in Fig. 4. It is to be noted that the kinetics of the reaction depends greatly on the molar ratio of Zn/ATP. Thus, at $\text{Zn/ATP} = 0.5$ the kinetics of the curves in Fig. 3 as

well as in Fig. 4 is of zero order, the experimental points falling into straight lines in each case, while at the ratios of 0.67 to 1.0 the curves in both figures appear to follow the kinetics of a reaction of first order, the per cent hydrolysis being independent of original concentration of ATP or of Zn.¹

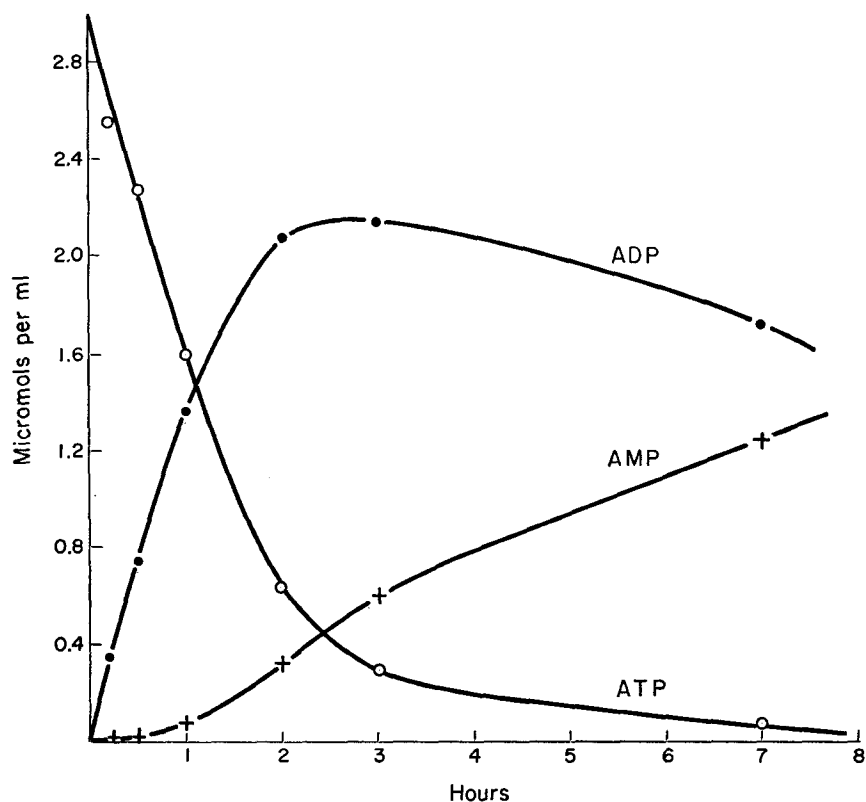


FIGURE 2. Course of hydrolysis of ATP by crystalline pyrophosphatase at 30°C in the presence of Zn by ion exchange on Dowex 1 columns.

Reaction mixture:

4 ml 0.1 M NH_4 acetate pH 7.0

4 ml 0.01 M ATP pH 7.0

2 ml 0.01 M ZnCl_2

2 ml H_2O

Warmed to 30°C + 2 ml cold 0.02 M NH_4 acetate containing 100 μg twice crystallized pyrophosphatase. One ml samples treated for ion exchange.

The zero order kinetics at the ratio $\text{Zn}/\text{ATP} = 0.5$ can be partly explained on the assumption that the rate of hydrolysis of ATP into ADP and PO_4 is proportional, first to the concentration of unhydrolyzed ATP, and second, to the ratio of concentration of zinc ions over concentration of unhydrolyzed ATP so that

¹ The molar ratio of $\text{Zn}/\text{ATP} = 0.5$ was conveniently used in assaying ATPase-Zn activities.

$$\frac{dp}{dt} = k(\text{ATP}_0 - P) \times \frac{\text{Zn}}{(\text{ATP}_0 - P)}$$

where P = free phosphate, ATP_0 = original concentration of ATP,

Hence $\frac{dp}{dt} = k \text{ Zn} = \text{constant } c$ (reaction of zero order kinetics)

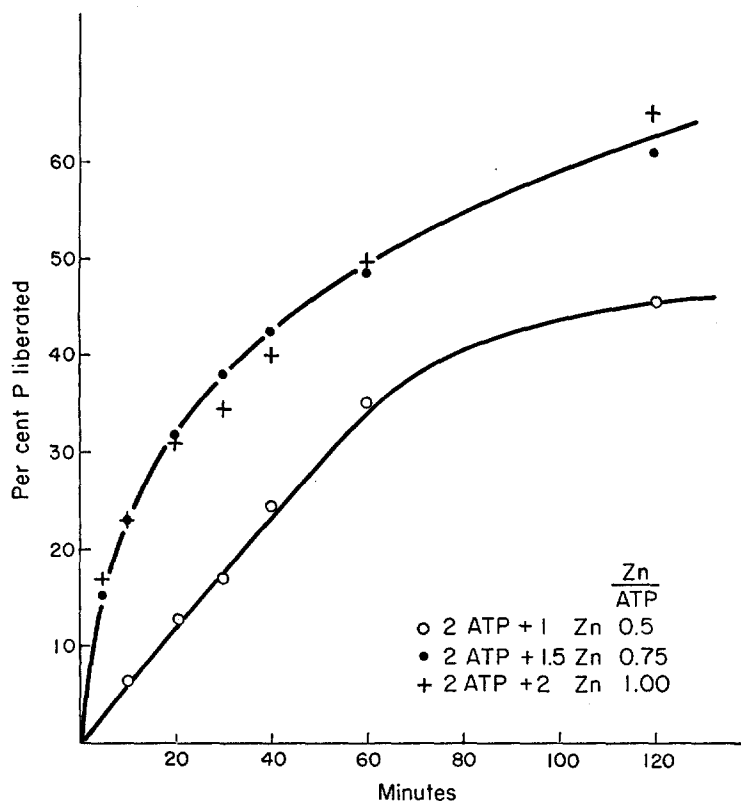


FIGURE 3. Effect of concentration of Zn on the kinetics of hydrolysis of ATP by pyrophosphatase at 30°C.

Reaction mixtures:

8 ml 0.1 M NH_4 acetate

2 ml 0.01 M ATP pH 7.0

1 or 1.5 or 2 ml 0.1 M ZnCl_2

2 ml cold 0.02 M NH_4 acetate containing 160 μg twice crystallized pyrophosphatase
 H_2O to 14 ml. One ml samples for inorganic P.

The assumption of direct proportionality between rate of hydrolysis and the ratio of Zn/ATP in the region of $\text{Zn}/\text{ATP} = 0.5$ to 0.65 is justified by an examination of Fig. 5 where a curve is given showing the relation between initial rate of hydrolysis of ATP and the ratio of Zn/ATP in the range of 0.3 to 1.0. The curve is S-shaped with a steep rise and nearly linear in the region of 0.5 to 0.8; at higher ratios, however,

the proportionality decreases fast so that at a ratio of $\text{Zn}/\text{ATP} = 0.9$ the rate of hydrolysis does not depend on the ratio of Zn/ATP any longer and the reaction follows the kinetics of first order.

5. *Effect of Magnesium Ions on the Rate of Hydrolysis of ATP by Crystalline Pyrophosphatase in the Presence of Zinc Ions* The present studies have confirmed

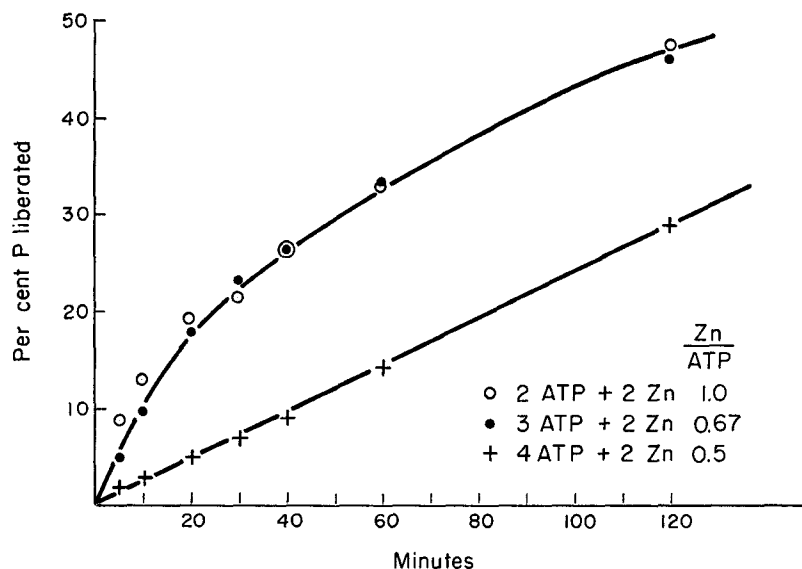


FIGURE 4. Effect of concentration of ATP on the kinetics of hydrolysis of ATP by pyrophosphatase at 30°C.

Reaction mixture:

6 ml 0.1 M NH_4 acetate

2 ml 0.01 M ZnCl_2

2 or 3 or 4 ml 0.01 M ATP pH 7.0

H_2O to 12 ml

2 ml 0.02 M NH_4 acetate containing 80 μg twice crystallized pyrophosphatase.

the findings of Schlesinger and Coon (2) that in addition to its powerful hydrolytic effect on inorganic pyrophosphate in the presence of Mg ions, crystalline pyrophosphatase exerts also a hydrolytic effect on ATP when used in relatively high concentrations, and when Mg is replaced by Zn as a catalyst in the reaction.² It was found, however, in the course of the present studies that in the presence of Zn in the hydrolysis mixture in the molar ratios of about 0.6 or less to the concentration of ATP, the addition of Mg ions does

² Several metal ions such as Mn^{++} , Co^{++} , Ni^{++} act as weak cocatalysts in both reactions. Also, Zn can be partly substituted for Mg in the hydrolysis of inorganic pyrophosphate. Magnesium, however, does not show any activating effect on the hydrolysis of ATP by crystalline pyrophosphatase in the absence of Zn or other metallic ions.

bring about considerable acceleration in the rate of hydrolysis of ATP by crystalline pyrophosphatase. Fig. 6 shows the effect on rate of hydrolysis of ATP on the addition of a constant amount of Mg to a series of solutions containing identical amounts of ATP but varying amounts of Zn ions. There is a considerable acceleration in the rate of hydrolysis brought about by Mg ions

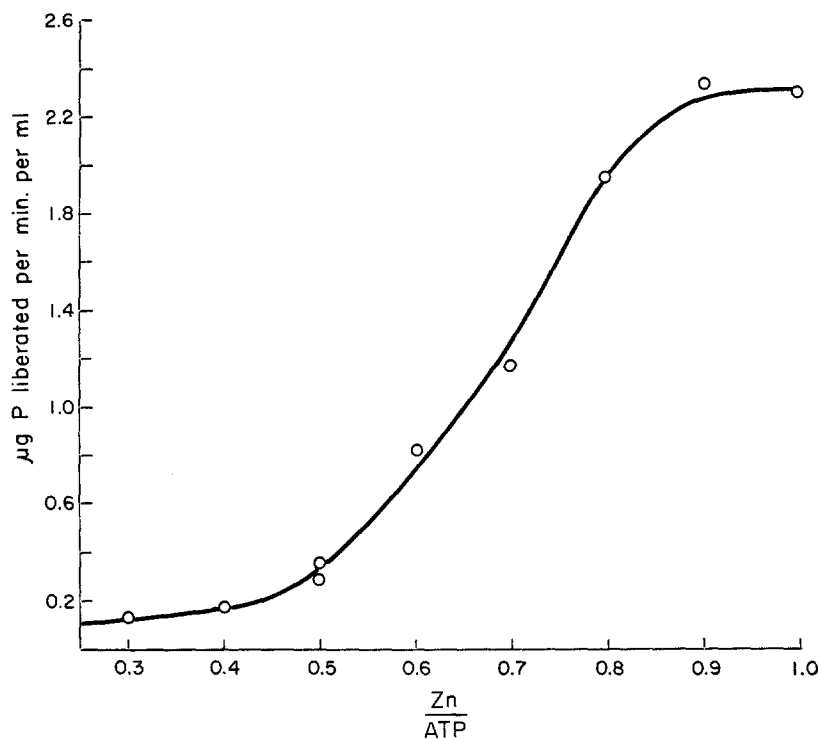


FIGURE 5. Relation between the rate of hydrolysis at 30°C of ATP by crystalline pyrophosphatase and the molar ratios of Zn to ATP.

Reaction mixture:

2 ml 0.1 M NH_4 acetate

2 ml 0.01 M ATP pH 7.0

Various amounts of 0.01 M ZnCl_2

1 ml 0.02 M NH_4 acetate containing 40 μg pyrophosphatase

H_2O to 7 ml

in the presence of concentrations of Zn insufficient to produce the maximum rate of hydrolysis; *i.e.*, in the region of ratios Zn/ATP of 0.2 to 0.55. There is no activation by Mg in the absence of Zn and there is a depressing effect by Mg ions on the rate of hydrolysis in the range of Zn/ATP of 0.6 or higher.

The effect of concentration of Mg on the kinetics of hydrolysis of ATP at constant Zn/ATP of 0.5 is shown in Fig. 7. There is nearly a direct proportionality between amount of Mg added and the increase in rate of hydrolysis,

the curves changing gradually from zero order kinetics in the absence of Mg to the kinetics of first order as the concentration of Mg is increased.

In the presence of a relative excess of Mg (Fig. 8), there is only a slight initial increase in rate of hydrolysis over that with Zn alone, or no increase at all, or even a definite reduction in the rate of hydrolysis.

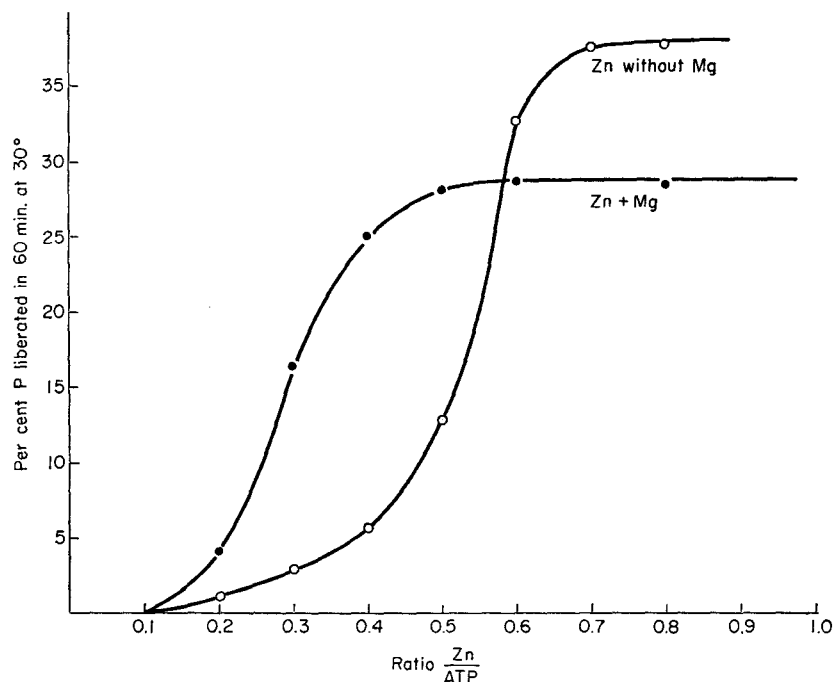


FIGURE 6. Effect of addition of Mg ions to Zn ions on the rate of hydrolysis of ATP by crystalline pyrophosphatase at 30°C. Reaction mixture same composition as in Fig. 5 except for substituting 1 ml 0.01 M MgCl_2 in each tube for 1 ml H_2O .

6. *The Course of Hydrolysis of ATP by Crystalline Pyrophosphatase in the Presence of Zn and Mg Ions* The effect of Mg ions on the course of hydrolysis when traced by means of ion exchange on Dowex 1 columns is shown in Fig. 9. The kinetics of the curves for ATP and ADP is about the same as shown in Fig. 7 where the rate of hydrolysis was expressed in terms of liberated free phosphate. The presence of Mg increases the rate of reaction considerably, the curves being of zero order kinetics in the absence of Mg and of first order in the presence of Mg.

The effect of Mg on the rate of hydrolysis of ADP by crystalline pyrophosphatase in the presence of Zn is small as compared with the effect on the hydrolysis of ATP. See Fig. 10.

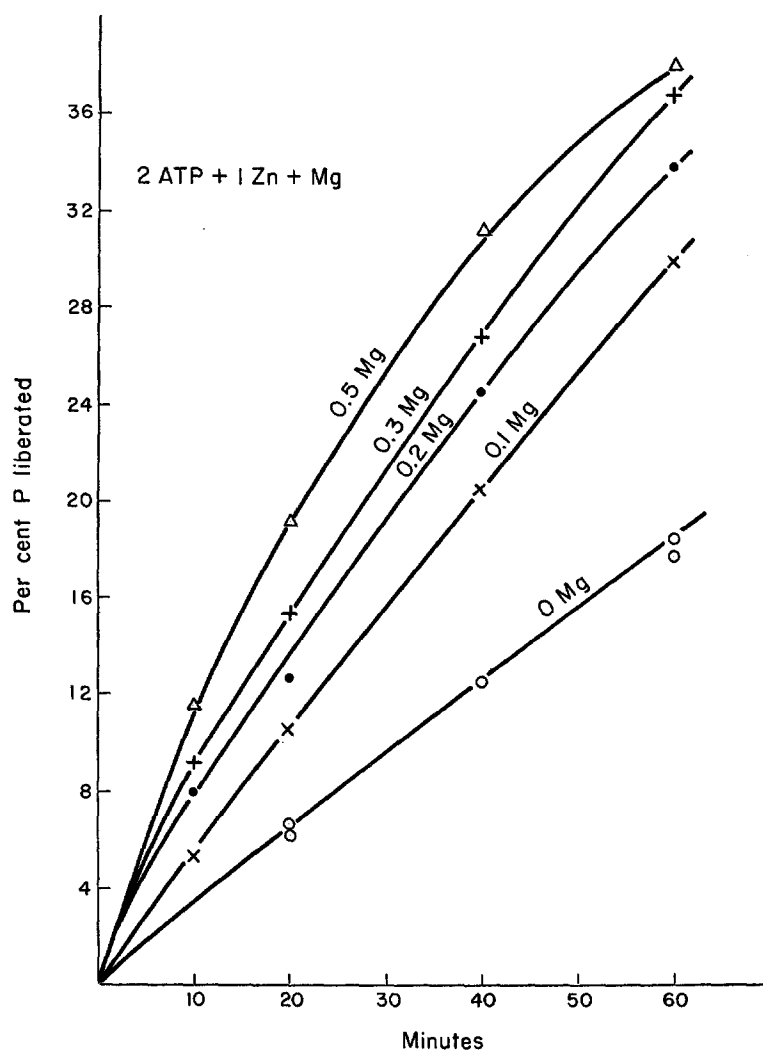


FIGURE 7. Effect of concentration of Mg ions on the kinetics of hydrolysis of ATP at constant Zn/ATP ratio of 0.5 and 30°C.

Reaction mixture:

4 ml 0.1 M NH_4 acetate

4 ml 0.01 M ATP pH 7.0

2 ml 0.01 M ZnCl_2

Various amounts of 0.01 M MgCl_2 ; H_2O to 12 ml

2 ml cold 0.02 M NH_4 acetate containing 80 μg pyrophosphatase.

7. *Effect of Various Ions on the ATPase-Zn Activity of Crystalline Pyrophosphatase*

Reaction mixtures:

0.4 ml 0.1 M NH_4 acetate pH 7.0

0.4 ml 0.01 M ATP pH 7.0

0.2 ml 0.01 M ZnCl_2

0.2 ml 0.01 M of various salt solutions mainly chlorides

0.2 ml 0.02 M NH_4 acetate containing 8 μg twice crystallized pyrophosphatase

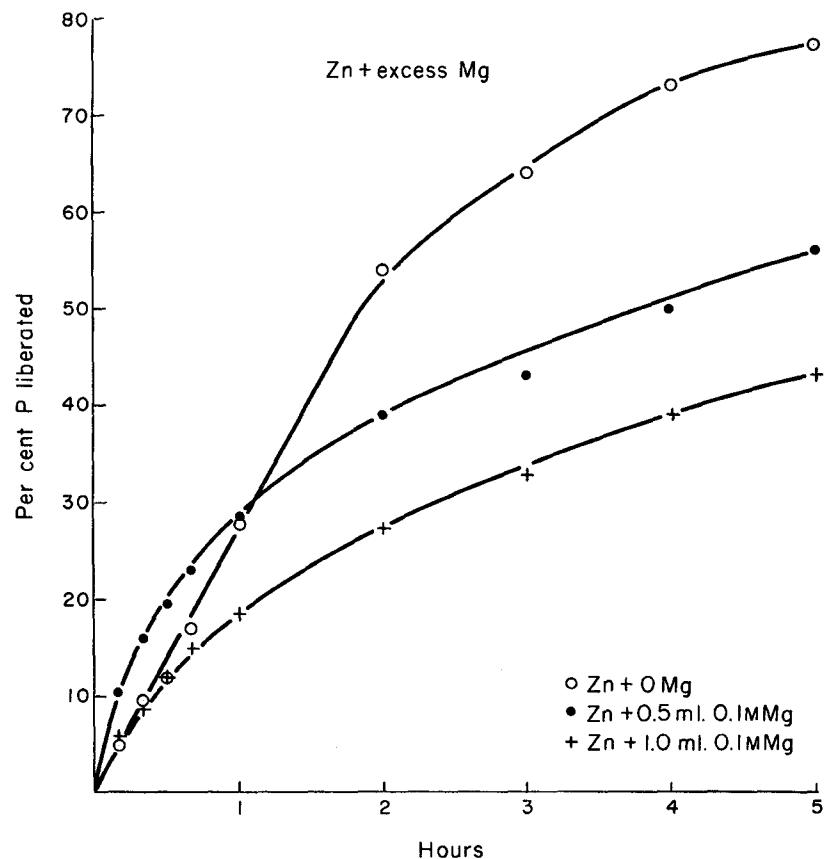


FIGURE 8. Antagonistic effect of excess of magnesium ions. Reaction mixture same composition as in Fig. 7, except for the use of 0.1 M MgCl_2 .

The mixtures were incubated for 30 minutes at 30°C, then mixed with 6 ml 0.17 N H_2SO_4 for inorganic phosphate determination. The results are given graphically on Fig. 11 which shows that in addition to Mg several other ions such as Mn, Ni, Co, La, exert an accelerating effect on the ATPase-Zn action of crystalline pyrophosphatase. It is to be noted, however, that these ions, even in the absence of Zn ions, bring about a significant rate of hydrolysis of ATP by the enzyme, while magnesium ion does not exert any cocatalytic effect on ATPase activity of pyrophosphatase in the absence of Zn ions, but it does affect the hydrolysis of ATP considerably in the presence of Zn.

CONCLUSIONS

The recent report by Schlesinger and Coon that crystalline yeast inorganic pyrophosphatase, in addition to its known ability to hydrolyze inorganic pyrophosphate in the presence of Mg ions, is also able to catalyze the hy-

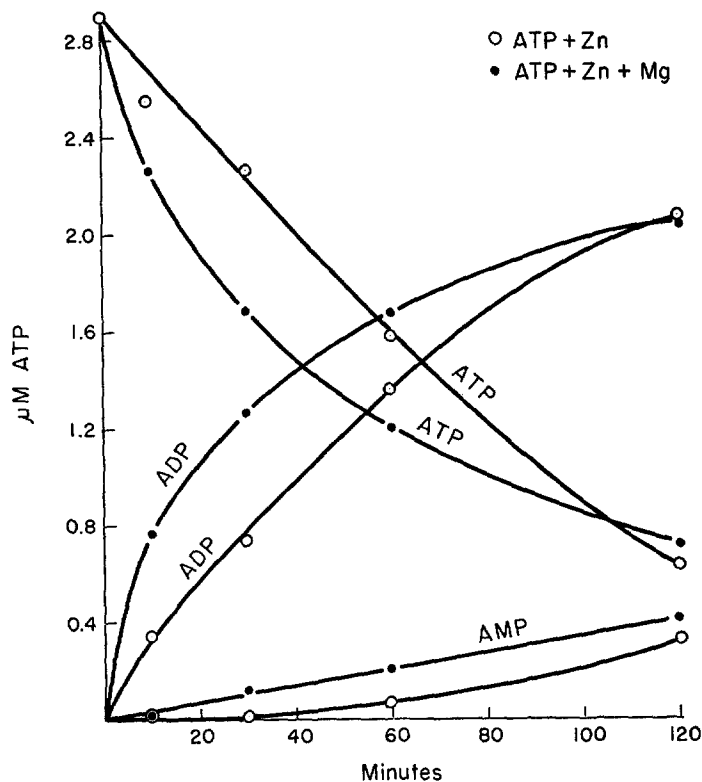


FIGURE 9. Effect of Mg ions on the course of hydrolysis of ATP traced by ion exchange on Dowex 1 columns. Reaction mixture of same composition as Fig. 2 except for substitution of 2 ml 0.01 M MgCl_2 for 2 ml H_2O .

drolysis of ATP and ADP in the presence of Zn ions was confirmed. A systematic study showed that the ratio of PPase-Mg over ATPase-Zn per milligram protein in various preparations obtained in the course of isolation of crystalline pyrophosphatase from baker's yeast was independent of the purity of the preparations used. These findings suggest strongly that the ATPase activity is not due to an accidental impurity which could be removed by the series of treatments, such as fractionation with ammonium sulfate and alcohol, autolysis, and repeated crystallization. Also, no separation of the

activities was noticeable in the course of a chromatographic treatment of a sample of crystalline pyrophosphatase with DEAE cellulose.

A study of the course of hydrolysis of ATP by crystalline pyrophosphatase

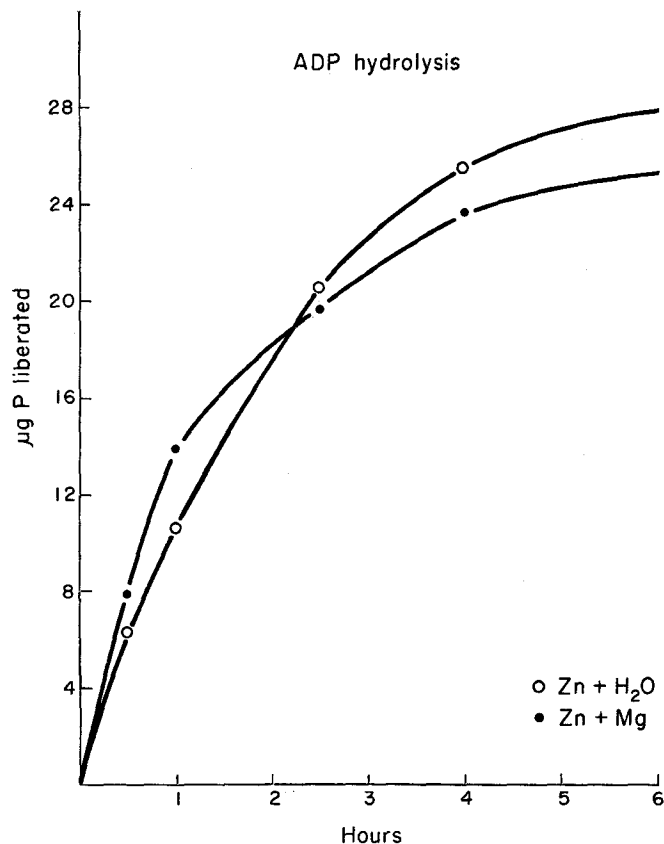


FIGURE 10. Hydrolysis of ADP at 30°C by crystalline pyrophosphatase in the presence or absence of MgCl₂.

Reaction mixture:

2 ml 0.1 M NH₄ acetate

2 ml 0.01 M ADP pH 7.0

1 ml 0.01 M ZnCl₂

1 ml H₂O or 1 ml 0.01 M MgCl₂

1 ml 0.02 M NH₄ acetate containing 40 µg pyrophosphatase

Samples of 1 ml for inorganic P.

in the presence of Zn, carried out with the aid of ion exchange on Dowex 1 columns, confirmed the finding of Schlesinger and Coon (2) that "the hydrolysis of ATP occurs primarily between the β - and γ -phosphate groups to yield ADP which is then converted to AMP." The first phase of hydrolysis proceeds at a greater speed than the second phase.

A study of the effect of varying the concentration of Zn and ATP on the rate of the first phase of hydrolysis showed that the kinetics of the reaction depends greatly on the molar ratio of Zn to ATP, being of zero order at $\text{Zn}/\text{ATP} = 0.5$ and of first order at Zn/ATP greater than 0.5.

The rate of hydrolysis of ATP into ADP in the presence of Zn ions is either accelerated or depressed on additions of Mg ions, depending on the ratio of Zn/ATP and on the relative concentration of magnesium ions as compared with the concentration of zinc ions in the hydrolysis mixture. Mg has a considerably accelerating effect in the presence of Zn ions in the ratios of Zn/ATP of 0.2 to 0.55, *i.e.* in the range of Zn/ATP insufficient to produce maximum rate of hydrolysis, and a distinct depressing effect of Mg

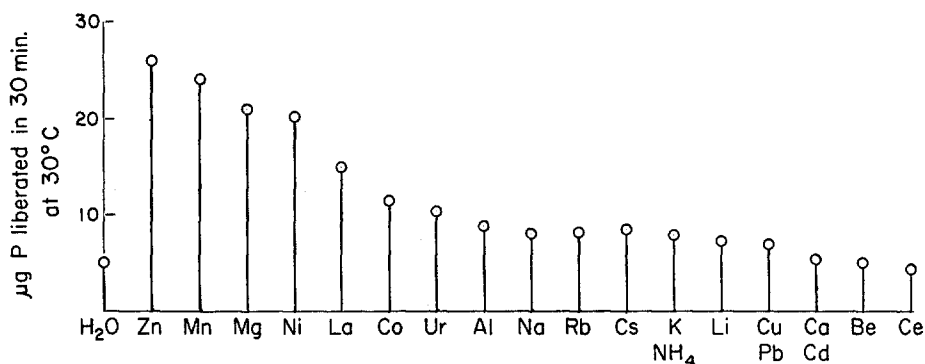


FIGURE 11. Effect of various ions on the rate of hydrolysis of ATP by crystalline pyrophosphatase in the presence of Zn.

in the range of ratios of Zn/ATP of 0.6 or higher, and no effect at all in the absence of Zn. A relative excess of Mg over Zn depresses the rate of hydrolysis of ATP even at the ratio of Zn/ATP of 0.5 or less.

The significant accelerating effect of Mg ions on the rate of hydrolysis of ATP by crystalline pyrophosphatase in the presence of concentrations of Zn insufficient to bring about maximum rate of hydrolysis, and also the effect of the Zn/ATP ratio on the kinetics of the $\text{ATP} \rightarrow \text{ADP}$ reaction, suggest strongly that both activities—ATPase and PPase—are manifestations of the same active group in the protein molecule of crystalline pyrophosphatase. The effect of the metal ions is on the substrates, the specific function of the Zn ions being to affect the strength of the pyrophosphate bonds in the ATP molecule, making it amenable to the action of relatively high concentrations of inorganic pyrophosphatase in the presence of Mg or Zn ions or both.

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